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**Trisomy 21 is a Cause of Permanent Neonatal Diabetes that is Autoimmune but not  
HLA Associated**

**Running title: Trisomy 21 causes autoimmune neonatal diabetes**

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the Supplementary file

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**Tweet (please include figure 1):**

Down syndrome can cause autoimmune diabetes before 6 months of age that is not HLA  
mediated, supporting that autoimmunity in DS can be caused by trisomy 21 and not HLA.  
From @mbjohnsonPhD and @ExeterMed colleagues #ExeterDiabetes

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## **ABSTRACT (160/200)**

Identifying new causes of permanent neonatal diabetes (diagnosis <6 months; PNDM) provides important insights into  $\beta$ -cell biology. Patients with Down syndrome (DS) resulting from trisomy 21 are 4 times more likely to have childhood diabetes with an intermediate HLA association. It is not known if DS can cause PNDM. We found trisomy 21 was 7 times more likely in our PNDM cohort than in the population ( $13/1522 = 85/10,000$  observed vs.  $12.6/10,000$  expected) and none of the 13 DS-PNDM cases had a mutation in the known PNDM genes which explained 82.9% of non-DS PNDM. Islet autoantibodies were present in 4/9 DS-PNDM patients but DS-PNDM was not associated with polygenic susceptibility to type 1 diabetes. We conclude that trisomy 21 is a cause of autoimmune PNDM that is not HLA associated. We propose that autoimmune diabetes in DS is heterogeneous and includes coincidental type 1 diabetes that is HLA associated and diabetes caused by trisomy 21 that is not HLA associated.

Permanent neonatal diabetes (PNDM) is diagnosed before the age of 6 months and a genetic diagnosis is possible for >82% of cases (1). Twenty-four causative PNDM genes have been identified (1–4) and 4 of these cause monogenic autoimmune PNDM that results from destruction of the  $\beta$ -cells very early in life (*FOXP3*, *IL2RA*, *LRBA* and *STAT3*). Identifying novel causes of autoimmune neonatal diabetes can provide key insights into the development of autoimmunity and can provide new targets for therapeutic intervention.

Down syndrome (DS) is caused by trisomy of chromosome 21 and has an incidence of 1:700 to 1:1100 live births (5). Large studies have shown childhood-onset autoimmune diabetes is 4 times more common in DS than in the general population, and has an intermediate HLA association (6). There have been three reported cases of DS with diabetes diagnosed before 6 months (7,8). However, it is not known whether DS was the cause of PNDM (DS-PNDM) or a coincidental finding in these patients. The aim of our study was to use our large international cohort of PNDM patients to assess whether DS can aetiologically cause PNDM. We assessed clinical phenotype, islet autoantibodies, and polygenic risk of T1D in patients with monogenic PNDM and DS-PNDM.

## **METHODS:**

### **STUDY POPULATION**

We defined permanent neonatal diabetes (PNDM) as diabetes diagnosed before the age of 6 months which is treated with continual insulin treatment. We studied 1522 DNA samples from two international collections of PNDM patients, 1360 recruited in Exeter and 162 recruited in Chicago. These either had a confirmed monogenic aetiology (82.9%, n=1262) or had been tested (and were negative) for all 24 known genes (17.1%, n=260). Clinical information was provided by the referring physician via a comprehensive referral form. This includes a section for the reporting of extra pancreatic clinical features.

### **GENETIC TESTING**

#### **Testing of the known genes**

All individuals diagnosed in the first 6 months of life were tested for mutations either by rapid Sanger sequencing of *ABCC8*, *KCNJ11* and *INS* or if no mutation was identified via targeted DNA sequencing through next generation sequencing (tNGS) of all 24 genes (1–4,9). This assay can detect single nucleotide variants, insertion-deletions, copy number variation and structural variation (9).

#### **Exclusion of Trisomy 21**

We used SavvyCNV (10,11) to screen for trisomy 21 in the PNDM and autoimmune-PNDM cohorts where tNGS was undertaken (n=445). This uses off-target reads from tNGS data to detect large copy number and structural variants.

#### **Type 1 Diabetes Genetic Risk Score**

The T1D genetic risk score was generated where there was sufficient DNA as previously described (12). Briefly, we genotyped single nucleotide polymorphisms (SNPs) tagging the top 10 risk alleles for T1D and summed their log transformed odds ratios before dividing by the total number of alleles to obtain a numeric score. None of the SNPs used are on chromosome 21. We used T1D-GRS from 1963 gold-standard T1D individuals (all diagnosed <17 years) from the Wellcome Trust Case Control Consortium as a representative samples for polygenic T1D (13). As part of the score generation, SNPs rs2187668 and rs7454108 were used to tag DR3 (DRB1\*0301-DQA1\*0501-DQB1\*0201) and DR4-DQ8 (DRB1\*04-DQA1\*0301-DQB1\*0302) alleles. These SNPs have been shown to be 98.6% sensitive and 99.7% specific for tagging DR3/DQ4-DQ8 (14). We used rs3129889 to tag HLA DRB1\*15 (15).

The T1D genetic risk score (T1D-GRS) was available for 13 patients with DS-PNDM, 458 individuals with a confirmed non-autoimmune monogenic aetiology (non-DS PNDM) and 40 individuals with a monogenic cause of autoimmune PNDM (autoimmune non-DS PNDM). There was no difference in clinical features between individuals with or without T1D-GRS.

## **ISLET AUTOANTIBODY TESTING**

Islet autoantibodies GADA, IA-2A and/or ZnT8A were measured either by local assays or from plasma on the sample received at the Exeter Clinical Laboratory using an automated ELISA based assay as previously described (16). We used >97.5<sup>th</sup> centile of controls (n=1559) to define positivity for autoantibodies.

## **STATISTICAL ANALYSIS**

The Mann-Whitney U and Kruskal-Wallis tests were used to compare continuous variables and the Fisher's exact test was used to compare categorical variables. Statistical analysis was undertaken in Stata14 (StataCorp, College Station, USA).

## **ETHICAL APPROVAL**

This study was approved by the Genetic Beta Cell Research bank, Exeter, UK and the University of Chicago, Chicago, IL, USA. Ethical approval was provided by the North Wales Research Ethics Committee, UK (IRAS project ID 231760) and the University of Chicago Institutional Review Board (IRB #6858, 15617B).

## RESULTS:

**Trisomy 21 is enriched in PNDM.** To assess whether DS is enriched in our PNDM cohort, we compared the observed frequency in the PNDM cohort to the expected frequency based on the population prevalence. Using clinical data obtained at referral we identified 13/1522 (0.9%) individuals with DS-PNDM in our international PNDM cohort. In all 13 individuals trisomy 21 was confirmed by karyotyping and analysis of tNGS data using SavvyCNV. We did not detect Trisomy 21 in any individual without a clinical diagnosis of Down syndrome. The observed frequency in our cohort was therefore 85/10,000 (95% CI 45-146), 6.7 fold higher than the expected population frequency of 12.6/10,000 (95% CI 12.4-12.8) ( $P=0.007$ ) (Fig. 1) (5).

**Patients with DS-PNDM do not have mutations in any of the 24 genes known to causes monogenic PNDM.** We next assessed the presence of known monogenic aetiology in patients with DS-PNDM. None of the 13 DS-PNDM individuals had pathogenic variants in the 24 known PNDM genes. In contrast, 1262 of 1522 (82.9%) of the non-DS PNDM had a monogenic aetiology (Fig. 2;  $P=1.4 \times 10^{-10}$ ). Taken together, these two results strongly support trisomy 21 as a cause of PNDM.

**Islet autoimmunity is common in DS-PNDM.** To further assess the underlying aetiology of DS-PNDM, we compared the clinical characteristics of the DS-PNDM patients with the PNDM cohort with a non-autoimmune monogenic cause (non-DS PNDM;  $n=458$ ) or monogenic autoimmunity (autoimmune non-DS PNDM;  $n=40$ ). We found that 44% (4/9) of the DS-PNDM individuals were positive for one autoantibody (all GADA; none IA2A or ZnT8A) with time from diabetes diagnosis to testing ranging from 4 months to 10 years (Table 1). This was similar to the autoimmune non-DS PNDM (46%, 13/28,  $P=1.0$ ) but higher



than the non-DS PNDM (21/293, 7%,  $P=0.004$ ) (Table 2). This supports an autoimmune aetiology in DS-PNDM.

The median age of onset of diabetes was similar in all three groups (Table 2). The birthweight of the DS-PNDM cohort was low (-1.23 SDs). This was similar to the non-DS PNDM individuals (-1.74 SDs,  $P=0.87$ ) and lower than those with autoimmune non-DS PNDM (-0.39 SDs,  $P=0.02$ ). Full clinical information for each subject with DS-PNDM and diabetes is given in table 1.

**DS-PNDM is genetically distinct from Type 1 diabetes.** To further investigate the aetiology of autoimmune diabetes in DS-PNDM we assessed polygenic risk for T1D (T1D-GRS) in DS-PNDM and compared it to both T1D and monogenic PNDM. T1D-GRS in individuals with DS-PNDM was similar to non-diabetic controls (Fig. 3, median T1D-GRS 0.61 vs 0.55,  $P=0.33$ ), non-DS PNDM (0.61 v 0.55,  $P=0.48$ ) and autoimmune non-DS PNDM (0.61 v 0.57,  $P=0.65$ ) but lower than the T1D control group (0.61 v 0.69,  $P=0.0001$ ). While 10/13 individuals with DS-PNDM carried a copy of either DR3 or DR4, none had the highest risk HLA DR3/DR4 genotype compared to 34% (666/1963) of T1D controls ( $P=0.006$ ; Table 1). Furthermore, 4/13(36%) DS-PNDM cases carried the HLA DRB1\*15 allele which is dominantly protective-against T1D compared to 2% (40/1963) of T1D controls ( $P=0.0001$ ). This is similar to non-diabetic controls who carry the protective HLA DRB1\*15 allele (1643/2938,  $P=0.27$ ). These data suggest that DS-PNDM is not HLA associated and is therefore unlikely to be very-early onset polygenic T1D.

## DISCUSSION:

Our study identifies trisomy 21 as a rare cause of autoimmune PNDM. We have shown that DS is 7-fold enriched in our PNDM cohort and none of the DS-PNDM individuals have a mutation in any of the 24 known PNDM genes. The antibody data supports an autoimmune aetiology in DS-PNDM that is not associated with increased polygenic susceptibility to T1D. DS-PNDM is rare within people with DS suggesting that trisomy 21 is a low penetrance cause of PNDM.

We found that PNDM caused by trisomy 21 can be linked to beta-cell autoimmunity as shown by the presence of islet antibodies in 4/9 cases (17). Islet antibodies are seen in HLA associated T1D and also in autoimmune non-DS PNDM which is not HLA associated (12). The proportion of patients with DS-PNDM that were autoantibody positive was similar to non-DS PNDM caused by monogenic autoimmunity. The islet antibodies are unlikely to due to maternal transfer of antibodies as of the 3 of the 4 GADA positive patients were measured after 9 months (18,19).

Our data suggest prenatal onset of beta cell dysfunction/destruction in DS-PNDM. We found that the birthweight of the DS-PNDM individuals was reduced (median z-score -1.23); this is lower than the birth weight seen in DS without PNDM (z-score boys: -0.47 girls: -0.07). Interestingly, the birthweight was similar to non-DS-PNDM patients whose lower birthweight is due to reduced insulin secretion *in utero*. Global immunological changes in individuals with DS have been identified including defects in T-cell regulation and thymus development that are evident in new-borns (20). These data taken together suggest prenatal onset of autoimmunity and pancreatic dysfunction.

We found that individuals with DS-PNDM do not have increased polygenic risk of T1D even though they develop diabetes before 6 months of age. This is contrary to T1D in which the

HLA association is increased with younger age of diagnosis (21). Furthermore, 4/13 (31%) individuals carry the DRB1\*15 haplotype which provides dominant protection against T1D and is very rarely seen in patients with T1D (40/1963; 2%). Two of these also carried DR3 or DR4, however inheritance with the DRB1\*15 allele nullifies the risk allele (22). The DS-PNDM cohort is of mixed ethnicities (6 Caucasian, 3 Middle Eastern, 3 Hispanic, 1 mixed), however population stratification does not account for the difference in HLA alleles between DS-PNDM cases and T1D; the HLA DR3/DR4 diplotype is strongly predisposing and the DRB1\*15 allele is strongly protective for T1D across these populations(23–25). These data support that DS-PNDM is not HLA associated.

A previous study showed that DS with diabetes had intermediate HLA association (7). The highest risk HLA diplotype - DR3/DR4 – was present in half as many DS diabetes cases compared to T1D (17% of DS diabetes, 38% of T1D, 3% of controls). We propose that this is explained by diabetes in DS in these studies reflecting a mixture of 2 subtypes of autoimmune diabetes, one caused by trisomy 21 related immune (not HLA associated) and the other is coincidental HLA associated polygenic T1D in which the trisomy 21 related immune dysregulation may not be playing as strong a role. A mixture of two aetiologies is also reflected by the observation that age of onset of diabetes in DS is biphasic, with a peak at one year and another at 10 years of age, the latter coinciding with non-DS T1D.

We have shown a 7 fold increase in the prevalence of diabetes before the age of 6 months in DS. Previous authors (6) have shown an increase of diabetes between 0.5-18 years of approximately 4 fold. The difference in prevalence means patients are far less likely to get diabetes before 6 months (7/100,000) compared to 0.5 -18 years (700/100,000).

A complex interaction between multiple genes on chromosome 21 may be responsible for autoimmunity in DS. The autoimmune regulator gene, *AIRE* , which is in the minimal region

for DS on chromosome 21, regulates the ectopic expression of tissue restricted antigens in the thymus to expose developing T cells to self-peptides; those that are strongly reactive are removed or reprogrammed (26). Mutations in *AIRE* cause Autoimmune Polyendocrine Syndrome Type 1 which commonly includes endocrine autoimmunity (27). *AIRE* has been shown to be aberrantly expressed in individuals with DS. A study of infant (0-6 months) thymus' removed during cardiac surgery found that *AIRE* mRNA and protein expression was elevated in DS cases (n=5) vs non-DS controls (n=5) (28). The mRNA expression of two genes under *AIRE*'s control in medullary thymocytes, *INS* (encoding insulin) and *CHRNA1* (encoding a subunit of muscle acetylcholine receptor) was also increased. Furthermore, this study found an increased overall number of medullary thymocytes expressing *AIRE*, which the authors propose could be linked to an effect on thymocyte turnover; *AIRE* is known to promote terminal differentiation in medullary thymocytes (29,30). *AIRE* has multiple complex roles in thymic function (including Treg selection, antigen expression, cell differentiation, antigen presentation and chemokine production (31)) and increased expression of *AIRE* was counterintuitively postulated to result in an alteration of the balance of these processes and therefore impaired thymic selection and reduced central tolerance resulting in increased autoimmunity.

Two larger studies of the DS thymus found that *AIRE* mRNA expression was reduced 2-fold in DS thymus', and that genes under *AIRE*'s control in medullary thymocytes had reduced expression (32,33). They found that the number of medullary thymocytes positive for *AIRE* was also reduced. Intriguingly, Gimenez-Barcons et al. found that all 3 copies of *AIRE* present in DS cases were equally expressed, albeit at reduced total levels, suggesting overcompensation by transcriptional repression of all alleles. The patients in these studies were older than those in the study by Skogberg et al. (2 months – 12 years), therefore a potential temporal relationship of *AIRE* expression in DS thymus, possibly linked to *AIRE*s

role in differentiation and turnover of thymocytes (29,30), could explain the difference. None of the cases were reported to have diabetes; however 11/19 reported by Gimenez-Barcons et al. had organ specific autoimmunity (hypothyroidism, Graves disease or celiac disease).

Additional genes contained in chromosome 21 have been linked to the increased incidence of autoimmune diabetes in DS. The *UBASH3A* gene, also on chromosome 21, has been associated (and the association replicated) with type 1 diabetes and has a role in the regulation of T-cells (34). *UBASH3A* down-regulates t-cell receptor induced NFκB signalling (35). NFκB signalling regulates multiple aspects of the innate and adaptive immune system including the expression of IL2 (36), a pleiotropic cytokine whose roles include the regulation of self-tolerance (37). Furthermore, a gene cluster containing four interferon receptors (*IFNAR1*, *IFNAR2*, *IFNGR2*, and *IL10RB*) is on chromosome 21 and it has been shown that increased interferon signalling is a hallmark of DS (38). RNA-sequencing experiments showed that interferon-related factors were consistently overexpressed in DS lymphocytes compared to controls. Increased interferon signalling has been implicated in multiple autoimmune diseases, including T1D (39–42).

To our knowledge this is the largest study of DS-PNDM. Study of additional individuals will provide further insight into the underlying mechanism of DS-PNDM. An interesting follow up would be to assess the T1D-GRS in an older cohort of DS individuals with diabetes to assess if genetic risk for T1D increases with older age at onset. Furthermore, we were unable to test all islet autoantibodies in all individuals near to diagnosis or perform immunophenotyping on their leukocytes. This would provide further understanding of the autoimmunity in DS-PNDM.

In conclusion, we have shown that trisomy 21 is a cause of PNDM. The underlying mechanism of the diabetes is likely to be due to autoimmunity against the beta cells. We

propose that diabetes in DS is heterogeneous and consists of a subgroup diagnosed very young (including PNDM) which is autoimmune but not HLA mediated and a second group that is similar to T1D in the non-DS population and has a strong HLA association. Extended genetic testing of known monogenic diabetes genes for individuals with DS-PNDM beyond the most common forms – activating mutations in *ABCC8* or *KCNJ11* – may not be needed.

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### **Author Contributions**

M.B.J, E.D.F, K.A.P and A.T.H. designed the study. M.B.J performed the genetic and statistical analysis, interpreted the data and wrote the first draft of the manuscript. K.A.P., E.D.F, S.W.G, L.R.L, S.E. and S.E.F. assisted with the interpretation of clinical information and contributed to discussion. M.N.W performed bioinformatics analysis. M.B.J, K.A.P. and A.T.H. wrote the manuscript which was reviewed and edited by all authors. A.T.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

### **Data and Resource Availability.**

The datasets generated during and/or analyzed during the current study are not publicly available due to patient confidentiality but are available from the corresponding author upon reasonable request.

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#### **Duality of interest**

No potential conflicts of interest are reported for this manuscript.

## FIGURE LEGENDS

**Figure 1 – Down syndrome is enriched in our PNDM cohort.** DS has a population prevalence of 12.6/10,000 (95% CI 12.4-12.8), whereas in our cohort of 1522 patients with neonatal diabetes we have 13 cases, equivalent to 85/10,000 (40.4-144.3);  $P=0.007$ ).

**Figure 2 – DS-PNDM is not caused by other known neonatal diabetes genes.** The 24 known neonatal diabetes genes account for 82.9% of cases while none of the 13 DS-PNDM cases had a mutation in a known gene ( $P=1.4 \times 10^{-10}$ ).

**Figure 3: the T1D-GRS in DS-PNDM.** Patients with DS-PNDM (n=13) had a lower score than T1D controls (n=1963), and their scores were similar to those with the known monogenic forms of non-DS PNDM and autoimmune non-DS PNDM (n=458 and n=40;  $P=0.52$ ) and non-diabetic controls (n=2938,  $P=0.33$ ). The central line within the box represents the median and the upper and lower limits of the box represent the interquartile range. The whiskers are the most extreme values within  $1.5 \times$  the interquartile range from the first and second quartiles.

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Patient	Sex	Ethnicity	Maternal age at birth	Patient Current age	Birthweight z-score <sup>a</sup>	Age diagnosed with diabetes	Treatment	HbA1c/% (mmol/L) <sup>b</sup>	Autoantibodies tested	Autoantibody status	Duration diabetes at antibody testing	T1D-GRS (centile of T1D) <sup>c</sup>	HLA DR genotype <sup>d</sup>	Other conditions
1	Male	Caucasian	ND	23 years	-0.79	7 weeks	Insulin	9.5 (80)	ND	ND	NA	0.37 (0.1)	DRB1*15/X	Hypothyroidism, ASD
2	Female	Hispanic	ND	14 years	-3.99	1 week	Insulin	8.1 (65)	ND	ND	NA	0.70 (53.5)	DR4/DR4	Hypothyroidism, hearing and vision problems
3	Male	Caucasian	33y	1 year	-1.02	2 days	Insulin	ND	GADA, IA2A, ZNT8A	Negative	1 week	0.69 (48.2)	DR3/DR3	Tetralogy of Fallot, macroglossia
4	Male	Caucasian	ND	28 years	ND	3 months	Insulin	8.5 (69)	GADA, IA2A, ZNT8A	GADA positive	10 years	0.52 (2.5)	X/X	Seizures
5	Male	Arabic	37y	1 year	ND	4 months	Insulin	8.8 (73)	GADA, IA2A, ZNT8A	GADA positive	4 months	0.66 (33.9)	DR3/DR3	-
6	Male	Hispanic	40y	9 months	-2.65	2 weeks	Insulin	5.7 (39)	GADA, IA2A, ZNT8A	Negative	2 months	0.64 (25.4)	DR4/X	Subclinical hypothyroidism, macroglossia, cardiac malformation
7	Male	Mixed (East Asian/Caucasian)	43y	20 years	0.80	3 days	Insulin	8.8 (73)	GADA	GADA positive	8 years	0.63 (20.6)	DR3/X	Hypothyroidism, coeliac disease exocrine insufficiency
8	Male	Arabic	ND	9 years	-1.23	8 weeks	Insulin	9.3 (78)	GADA, IA2A,	GADA positive	5 years	0.37 (0.1)	DRB1*15/X	Macroglossia
9	Female	Turkish	ND	15 years	ND	3 weeks	Insulin	8.1 (65)	GADA, IA2A	Negative	1 day	0.61 (14.3)	DR3/X	Hypothyroidism
10	Female	Hispanic	ND	Died aged 7 months	-3.30	3 days	Insulin	6.6 (49)	ND	ND	NA	0.49 (0.9)	DR4/DRB1*15	-
11	Male	Caucasian	35y	6 years	-0.84	1 day	Insulin	6.6 (49)	GADA, IA2A, ZNT8A	Negative	5 months	0.48 (0.8)	DR3/DRB1*15	Transient hypothyroidism, VSD
12	Male	Caucasian	ND	17 years	0.19	18 days	Insulin	9.8 (84)	ND	ND	ND	0.69 (48.2)	DR4/X	Seizures, hypothyroidism, frequent infections (low IgM), hepatic abscess
13	Male	Caucasian	ND	1 year	-1.85	Birth	Insulin	7.0 (53)	GADA, ZnT8A	Negative	ND	0.68 (44.5)	DR4/DR4	Hyperthyroidism, bowel obstruction, speech and vision problems

**Table 1: Features of the Down syndrome associated neonatal diabetes cohort.** ND – no data. a – z-score adjusted for sex and gestational age. b – latest HbA1c reported by clinician. c – Type 1 diabetes genetic risk score based on top 10 risk alleles; centile of T1D refers to the centile of the type 1 diabetes control population that this score corresponds with. d – ‘X’ denotes any HLA-DR haplotype other than DR3, DR4 or DRB1\*15. ASD – atrial septal defect. VSD – ventral septal defect

Characteristic	DS-PNDM n=13	Non-DS PNDM n=458	Autoimmune non-DS PNDM n=40
Age diagnosis diabetes (weeks)	2.3 (0.4, 7.5)	7 (3, 12)	4 (1, 13.5)
Female %	23% (3/13)	43% (197/458)	12.5% (5/40)*
Birthweight z-score†	-1.23 (-2.65, -0.84) [n=10]	-1.74 (-2.55, -0.82) [n=378]	-0.39 (-1.06, 0.29)‡
≥1 Autoantibody positive§	4/9 (44%)	21/293 (7%)	13/28 (46%)
T1D-GRS¶	0.61 (0.49-0.66)	0.55 (0.51-0.61)	0.57 (0.52-0.62)

**Table 2: Comparison of Down syndrome associated neonatal diabetes (DS-PNDM) with monogenic non-autoimmune forms of neonatal diabetes (Non-DS PNDM) and monogenic autoimmune neonatal diabetes (autoimmune non-DS PNDM).** \*Low due to males with X-linked IPEX syndrome (n=25/40) †adjusted for sex and gestational age. ‡DS-PNDM v autoimmune non-DS PNDM  $P=0.02$ . §One or more positive titre for GADA, IA2A or ZnT8A. ||DS-PNDM v non-DS PNDM  $P=0.006$ . ¶Type 1 diabetes genetic risk score; centiles based on T1D controls. Age of diabetes diagnosis, birthweight Z-score and T1D-GRS are given as median with inter-quartile range in parenthesis. Other than where indicated, data were similar for all cohorts ( $P>0.1$ ).

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